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VOLUME I NUMBER 2

STAIN TECHNOLOGY

APRIL, 1926

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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

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Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered, with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

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STAIN TECHNOLOGY

VOLUME I APRIL, 1926 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

Under this heading it is planned to include in each issue of this journal, or at least in frequent issues as space allows, various information of a more or less general nature concerning the findings of the Commission and the conclusions it has drawn from them. The editor assumes all responsibility for the views expressed in these reports. They may, in fact, be regarded as editorials; and yet it is ordinarily planned to obtain comments and criticism from other members of the Commission before publishing.

For the first year a series of articles is planned dealing, first with the progress made in the general methods of standardization, and secondly with the results obtained concerning the different groups of stains, one by one. In each case it is intended to discuss the situation as it existed in 1920 and then to show the steps that have been taken to improve matters.

METHODS OF STANDARDIZATION

When the work was begun, it was recognized that different lots of stains differed in their performance. Why some were good and others poor was not known. It was quickly learned, moreover, that the performance of the dyes as stains bore no relation to their purity. Reliable methods of standardizing were, in fact, entirely lacking. The chemistry of many of the stains was only vaguely understood; and the spectrophotometer (which had been found of great value in the analysis of other dyes) had not then been applied to biological stains, and its findings in regard to them, therefore, could not be properly interpreted.

The first step in the development of methods was taken thru the cooperation of the Color Laboratory of the Dept. of Agriculture (then under the direction of Dr. J. A. Ambler) in the form of an investigation of methylene blue. Methylene blue was selected because the reports already obtained from biologists showed more clear-cut distinctions between good and poor samples in the case of this stain than in the case of any other. The samples already tested by the biologists were submitted by the Color Laboratory to as complete chemical analysis as methods then known permitted, in the hopes of finding some correlation between performance and chemical data. Altho much time was given to this work, it was unfortunately found

that no such correlation existed. The only encouraging feature in this early work was a tendency for the good samples to show different absorption spectra from the poor ones when tested with a spectrophotometer.[1] In other words it was noticed that the relative light absorption in the different parts of the spectrum was slightly different in the case of these two classes of samples.

This difference in the light absorption of the good and of the poor stains was not constant, but it was the only promising line of attack suggested by these chemico-physical studies. It has proved a very valuable means of learning the identity of any sample and of explaining peculiarities in stain behavior; much stress is, therefore, laid upon the method at the present time. It was hoped at the outset that the spectrophotometric analysis might be used as a primary means of characterizing satisfactory stains; but the method has not yet been developed sufficiently for this to be possible.

In other words it has proved impossible to use chemical tests in the standardizing of stains or to depend wholly upon spectrophotometric analysis. The only other method of standardization that suggested itself was by means of biological tests. The only practical way of using such tests seemed to be to require the manufacturer of stains to submit to the Commission a sample of every batch they planned to put on the market, for distribution to experts in the use of that stain who could test its actual performance for staining microscopic preparations. This method of attack has, therefore, been combined with the spectrophotometric analysis of the samples. Specifications have been drawn up for the most important biological stains[2] and it is expected that samples submitted to the Commission shall conform with these specifications.

The specifications state a minimum dye content permissable, require conformance with a certain type of absorption curve, and insist that the sample shall give satisfactory results in the hands of experts in its use. Samples found to meet such requirements as these are sold under the certification label of the Commission, with which biologists in this country are now pretty well familiar.

This method of attack is really quite similar to the method by which textile dyes are standardized at present. Altho textile dyes have been on the market much longer than stains, and altho dyeing is a much simpler art than staining for microscopic study, it has not yet proved possible to specify textile dyes either by chemical analysis or by absorption spectra. Before purchasing a dye a textile dyer wants

[1]For a description of the spectrophotometer and its applications to dye analysis, see Conn, Biological Stains, Geneva, N. Y., 1925, Chap. III, pp. 25–31.

[2]See Conn, Biological Stains, Geneva, N. Y., 1925, pp. 132–137.

to see what it will actually do when applied to the fabric for which it is to be used. Such being the case, it is not surprising that the microscopist has to fall back on the same method. It is, nevertheless, much less satisfactory than would be a standardization based on physico-chemical analysis. It is particularly unfortunate that this type of standardization has given little help to workers in government laboratories who are forced to buy by the methods of procurement specified by law. The failure of the commission's specifications to assist government laboratories is well explained in the recent article by R. W. French.[3]

It is generally conceded, nevertheless, that the present supply of stains is much superior to that available in 1920; and it is felt, therefore, that the method of standardization adopted, altho not ideal, has been yielding results.

Standardization of Staining Solutions

It might be possible to secure uniformity and high quality in all the stains put upon the market without assuring constancy in the results obtained in actual use. This is because to assure absolute agreement in different laboratories the formulae for staining solutions as well as the stains themselves must be standardized. Two earlier reports of the Stain Commission have called special attention to certain weaknesses in staining formulae. The first of these two reports[4] called attention to the variation possible in staining solutions when made up on the basis of a definite weight of dry dye, when the actual dye content of the dye in question was not known. The second report[5] showed that not much greater constancy in the formulae could be secured if they were based upon the use of a definite volume of a saturated alcoholic solution of the dye. Solutions of the latter type, it was pointed out, possess a certain advantage over those that call for a definite weight of dry dye of unspecified strength; but the greater solubility of many dyes when pure than when mixed with certain mineral salts makes it impossible to secure uniformity by relying upon saturated solutions. It was stated, therefore, that the only way of securing uniformity is to specify exact weights of dye *on the basis of the actual dye content* of the sample used.

Attention is now called to the fact that staining formulae require standardization in other particulars as well. How often the following type of formula is seen:

---

[3] R. W. French. Standardization of biological stains as a problem of the Medical Department of the Army. Stain Tech. 1, 11–16.

[4] The preparation of staining solutions. Sci. 57, p. 15, 1923.

[5] Dye solubility in relation to staining solutions, Sci, 57, p. 638, 1923.

Alc. sol. fuchsin	1 part
Water	10 parts

What does this formula mean? What strength alcoholic solution? What strengh alcohol? What fuchsin? What strength fuchsin? Are the proportions 1 to 10 measured by volume or by weight?

It is very plain that the authors of staining formulae ordinarily do not know the answer to some of these questions themselves, while in other cases they assume that the answers are obvious to everyone. In many cases, indeed, they are obvious to anyone who has had much experience in preparing staining solutions; but they are hardly so to the uninitiated. It is much more accurate and not much more difficult to put this formula in the following form (assuming the bottle of stain bears the dye content of the sample, as is now the case with the most reliable stains put on the market):

Sat. Sol. Basic fuchsin (dye content 88%) in 95% ethyl alcohol	1cc.
Distilled water	10cc.

It would be still more satisfactory if the author of such a formula, before publishing it, determined the amount of basic fuchsin in 1 c.c. of his saturated solution (which we will assume to have been 0.06 g.) and then computed the amount of actual dye present, which in the case just mentioned would have been 0.053 g. Knowing these facts, his formula could be published in the terms of a definite weight of dry dye and of actual dye present in the stain used, somewhat as follows:

Basic fuchsin (88%)	0.6	g.
	(*representing*	*0.53 g. actual dye*)
Ethyl alcohol (95%)	10.	cc.
Distilled water	100.	cc.

This type of formula is recommended to all those publishing new staining methods.

Equal lack of clearness is often encountered in directions for preparing the solutions. A statement such as the following is sometimes found in connection with a staining formula: "Boil the dye with dilute sulphuric acid." It can hardly be regarded as obvious to anyone how dilute the acid should be or how long the boiling should be continued and yet in the case of a delicate chemical reaction such as might be involved, variation in either of these points might make considerable difference.

Obviously the Commission on Standardization of Stains cannot standardize staining formulae, as it does not control the publication

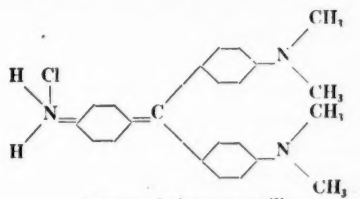
of the journals and books in which they appear. Cooperation must be secured on the part of investigators publishing new formulae and of text-book writers who are republishing old ones; also on the part of stain manufacturers securing statements of the dye content on the label. Stain manufacturers have already begun cooperating in the latter respect; and the first step, therefore, in standardizing formulae has been taken. The second step will be taken when it becomes the custom of those publishing new formulae to put them in the form recommended above. The third step, the inclusion of these accurate formulae in text-books, will follow more slowly; but not until it is done will the actual standardization of stains be complete.

Grades of Gentian Violet

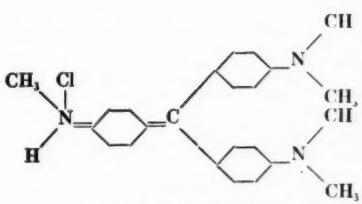
In the specifications drawn up by the Commission for gentian violet,[6] the following statement occurs:

"Gentian violet.... must be either penta-methyl or hexa-methyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B."

The exact meaning of this is perhaps not evident on the surface; and yet it is a matter of significance enough so that biologists should understand it. It will be recalled that methyl violets are varying mixtures of the following three compounds:

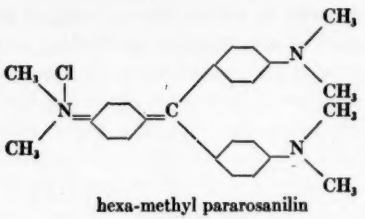


tetra-methyl pararosanilin



penta-methyl pararosanilin

[6]Conn, H. J., Biological Stains. Geneva, N. Y. 1925. See p. 135.



hexa-methyl pararosanilin
(crystal violet)

The bluer shades of methyl violet are denoted by symbols such as methyl violet 2B, 3B, etc. In these bluer shades the second two compounds predominate, namely penta- and hexa-methyl pararosanilin. Hexa-methyl pararosanilin alone is still bluer and is known as crystal violet.

Taking these facts into account it is evident that the definition of gentian violet adopted by the Commission means that it must be either crystal violet or one of the bluer methyl violets. This definition was adopted in accordance with the earlier results,[7] indicating that histologists and bacteriologists ordinarily obtained best results when crystal violet or methyl violet 2B was used as gentian violet in the various technics under investigation. The Commission would prefer that users order one or the other of these two products by the correct name and thus know what they are employing; but inasmuch as there is a demand for gentian violet which will undoubtedly continue for a good number of years, the stain dealers have to sell something under that name. Hence the above definition was adopted as one that would tend to give a high-grade article without excluding any of the types that might be preferred for certain purposes.

This definition does not secure uniformity, however. Some manufacturers prefer to sell crystal violet under the name of gentian violet and thus carry only one product for both purposes. Of those supplying gentian violet of this sort, samples have recently been submitted from the Empire Biochemical Co., Hartman-Leddon Co., and the National Aniline and Chemical Co. One other manufacturer, namely Coleman and Bell, sells as gentian violet one of the more highly methylated methyl violets. This latter product is of a noticeably redder shade than crystal violet. (Coleman and Bell also carry a crystal violet, designated as such.)

It is impossible to pronounce any general verdict as to the relative merits of these two grades of gentian violet. The bluer shade is preferred by some, the redder by others. The preference thus ex-

[7]Com. on Bact. Technic. An Investigation of American Gentian Violets. J. Bact. 7, 529–536. 1922.

pressed does not depend wholly on the purpose for which used. This was proved in the case of two recent samples sent out to different investigators, one obtained from the National Aniline and the other from Coleman and Bell, and representing the two types as above mentioned. Two investigators, in comparing them, employed almost exactly the same technic, and both spoke of the reddish shade of the Coleman and Bell product; but whereas one investigator expressed a decided preference for this sample, the other was equally firmly in favor of that from the National Aniline.

Hence it seems important that both grades be kept on the market and that users know where to obtain each. One might be designated "gentian violet, bluish," the other "gentian violet reddish." This is not yet done; but users can be certain which they have, if they are using certified stains, by noticing the Colour Index number; the redder stain is marked C. I. No. 680, the bluer C. I. No. 681. (That is to say, the more highly methylated dye has the higher Colour Index No.)

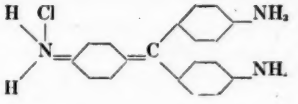
Recent Information Concerning Basic Fushsin

Of the various uses to which basic fushsin is put in the biological laboratory. it has proved particularly necessary to take two different procedures into account in passing upon the merits of any sample of the dye under investigation. These two are the Ziehl technic for staining the tubercle organism and the Endo technic for distinguishing between the members of the colon-typhoid group of bacteria. Both of these procedures are especially referred to in the specifications for basic fuchsin already drawn up by the Commission.[8]

Of these two procedures the former is an easily interpreted test. It proves possible to use nearly any basic fuchsin in this technic, altho with varying degrees of success, for differences are noticeable in the shade of the tubercle organisms when stained by it and in the intensity with which they are stained. Verdicts from different sources as to the relative merits of the different fuchsin samples differ; but the differences in opinion seem to depend partly upon whether the individual performing the test desires a bright red or a purplish stain.

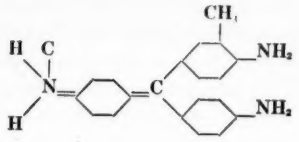
It will be remembered that basic fuchsins are ordinarily mixtures in different proportions of three primary types: namely,

pararosanilin



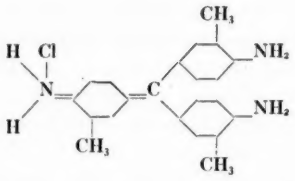
[8]A Report on Basic Fuchsin. Science, **54**, 556–557. H. J. Conn, Biological Stains. Published by the Commission, Geneva, N. Y., 1925. See p. 133.

rosanilin



. . .

and new fuchsin



Pararosanilin and new fuchsin are often encountered in comparatively pure state; rosanilin is ordinarily obtained mixed with pararosanilin but is occasionally put on the market free from the latter. Theoretically another primary basic fuchsin exists, namely the compound between rosanilin and new fuchsin, in which two of the benzene rings are methyl-substituted. This compound may possibly exist in some of the stains offered the biologist; but its presence has not yet been definitely ascertained.

In the article above mentioned (Sci. **60**, p. 387) the following statement was made:

"The results of the investigation showed quite plainly that, altho pararosanilin is ordinarily satisfactory as a bacterial stain and for other staining purposes, it is not usually satisfactory in the Endo medium. One sample was found which gave good results when used for the latter purpose, but just why it proved better than the others has not been learned. The best sample for all purposes, as judged by the five reports received from different investigators, was the sample of rosanilin without pararosanilin. Almost equally good, however, were some of the samples of new fuchsin."

Further investigations make it necessary to revise this statement. The samples which were assumed in this early work to be new fuchsin were not actually that dye. The conclusion as to their nature was based upon spectrophotometic examination, and it proves to have been a mistake. Actual new fuchsin samples have since been obtained and have been found to be distinctly different. That type of fuchsin which was considered at first to be new fuchsin is an easily recognizable type which is still on the market; (see Table 1, first footnote) but its chemical nature is not yet known. It is not a rosanilin and is nearer a new fuchsin chemically than it is to a para-

rosanilin; its identity is being investigated at the present time, and it is hoped that the investigations will bring out facts of some value.

This being the case, the statement quoted above that some samples of new fuchsin are almost as good for the Endo medium as the best sample previously investigated is incorrect. As a matter of fact, true new fuchsin proves absolutely unsatisfactory for this purpose. The fuchsin samples that actually prove best in the Endo medium are either pararosanilin, rosanilin, or of the unknown type just mentioned. Strange to say, the good samples for this purpose are not necessarily of the same chemical type; while on the other hand, of samples apparently identical chemically some may prove good and others unsatisfactory.

Meanwhile a test has been devised by French[9] for distinguishing between the samples that are satisfactory for the Endo medium and those that are not. The details of the method are to appear shortly in French's paper; enough here to state that it depends upon the principle of adding a measured amount of sodium sulphite to a weak fuchsin solution, determining the amount needed to effect decolorization, and then noticing the amount of a dilute formalin solution necessary to restore the color. In practice this proves to give results nearly parallel with those obtained in actual use.

This is shown in Table 1. This table compares the results by French's test with those obtained in actual use, employing a series of fourteen different basic fuchsins of various chemical nature. It will be seen that there are five different samples that were classed as excellent by French's technic, one (149) a para-rosanilin, two (142 and 148) rosanilin, the other two (42 and 43) of the unknown chemical type above referred to. In the case of each of these samples except 148, two reports (each from a different investigator) have been received, and there is not perfect agreement between the two reports in each case; but the average standing of each sample is "Good." There are on the other hand five samples (146, 52, 141, 144, and 147) which proved unsatisfactory as judged by French's test. They, too, are of various different chemical types. In the case of all of these except 141 and 144 the average of the two reports in the Endo medium was only "Fair." Samples 141 and 144, on the other hand, proved satisfactory in actual use. In spite of the disagreement in the case of these two samples of pararosanlin acetate, it is apparent that the agreement between the results of French's test and the average of the two reports received is closer than that between the two reports themselves. Hence it is regarded as a fairly

[9]The Journal of Laboratory and Clinical Medicine 11, 571, 1926.

TABLE 1. COMPARISON OF FUCHSIN SAMPLES FOR USE IN ENDO MEDIUM.

Sample Number	Chemical Nature	French's Formalin Test†				Test in Actual Use			
		Decolorization with sulfite		Restoration of color with formalin		Conclusion	First report	Second report	Ave.
		No. of cc. used	Success	No. of cc. used	Success				
146	New fuchsin	1.7	Poor	2.0	None	U‡	U	G	F
41	?*	1.5	Excel.	1.5	Good	G	G	F	G–
42	?*	1.0	Excel.	1.0	Good	E	G	G	G
43	?*	1.0	Excel.	1.0	Good	E	G	G	G
51	?*	1.2	Fair	1.0	None	U	F	F	F
31	?*	0.7	Good	2.0	Fair	F	U	G	F
32	?*	1.0	Good	1.5	Good	G	G		
142	Rosanilin	0.5	Excel.	1.0	Good	E	F	G	G–
148	Rosanilin	0.7	Good	1.0	Good	E	G		
141	para-rosanilin acetate	1.2	Good	2.0	Poor	U	G	F	G–
144	para-rosanilin acetate	1.2	Fair	2.0	None	U	G	G	G
149	para-rosanilin acetate	0.5	Excel.	1.0	Excel.	E	E	G	G†
143	para-rosanilin chloride	0.8	Excel.	1.5	Good	G	F	E	G
147	para-rosanilin chloride	1.8	Poor	2.0	None	U	F	F	F

*These five samples are of the unknown type, similar to new fuchsin, referred to on p 56.

†Figures state the number of c.c. of 1.5% aqueous sodium sulfite needed to decolorize 10 cc. of 0.05% aqueous solution of dye, and the number of c.c. of 0.5% formalin (i.e. 0.2% formaldehyde) necessary to restore color.

‡The significance of these letters is: E, excellent; G, good; F, fair; U, unsatisfactory.

accurate means of predicting the performance of a sample in the Endo technic.

Unfortunately up to the present time few samples have been found that are very satisfactory both for staining and for use in the Endo medium. Those samples of pararosanilin and rosanilin which prove good for the latter purpose, do not ordinarily give a satisfactory stain of the tubercle organism. Some companies are now, therefore, putting out two grades of fuchsin, one for staining, the other for the Endo medium. This is undesirable; and it is hoped that before long it will be possible to standardize this stain by assisting the manufacturers to put on the market one grade satisfactory for both purposes. The most promising samples for both purposes yet encountered are

those of the unknown type represented by the five samples listed in the table immediately following the new fuchsin sample 146.

At present the conclusions from this investigation are these: New fuchsin is a very good stain for the tubercle organism, but is entirely unsatisfactory for the Endo medium. Rosanilin is only fairly satisfactory as a stain for the tubercle organism, but is a good Endo fuchsin; while pararosanilin is sometimes extremely satisfactory for the Endo technic, sometimes entirely unsatisfactory (the acetate seeming better than the chloride), but is not very good in the Ziehl technic. There is, however, another type of fuchsin, whose chemistry is not yet understood, which generally proves very good for both purposes. The latter is at present being investigated.

Geneva, N. Y. H. J. Conn.

A METHOD FOR THE STAINING OF BACTERIAL FLAGELLA

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ABSTRACT.—**A modification of the Loeffler method of staining bacterial flagella is proposed. The chief points of the modification are: The cultures are inoculated into distilled water after two successive daily transfers on agar slants, and the distilled water cultures are incubated at optimum temperature for from 48 to 72 hours. The mordant (tannic acid, ferrous sulphate, basic fuchsin) is allowed to stand 18 to 24 hours before use, and then cleared by centrifuging or filtering. An anilin water fuchsin is used as a stain. No heat is used for either mordanting or staining; but both mordant and stain are allowed to act on the preparation for 15 minutes. The writer finds the method admirably adapted for use in class work, where nearly 100 per cent success has been obtained except in the case of some three or four species of bacteria that are especially difficult to stain.**

So-called staining of bacterial flagella is generally a bugbear to bacteriologists, especially to instructors who use this as a laboratory exercise for students.

Several methods have been reported as giving very excellent results in the hands of some individuals. These same methods, however, in the hands of others, even skilled technicians, often give poor or worthless results.

The writer has frequently attempted flagella staining by several methods, employing the most painstaking technic and giving fullest attention to details. The net results of several hundred trials have been possibly three or four successful preparations. Perhaps one student in a division of fifty attained reasonable results and, strange to say, that student often was one of the poorest technicians in the class. To sum up, it appears that successful flagella staining can be accomplished only by those who are peculiarly endowed for such work. Luck is of course quite a factor.

There has been developed in this laboratory a method for flagella staining which, when applied to certain organisms, has almost invariably given success. During the past two years practically every member of the class has succeeded in making one or more successful flagella stains without any particular effort other than following simple directions in detail.

Because of this unusual success in technic, the writer has been prevailed upon to add the details of this method to its many predecessors, with the hope that others may be able to obtain equally satisfactory results.

The method, which is a modification of Loeffler's,[1] is as follows:

A. Preparation of culture.

A fresh agar slant having an appreciable amount of condensation water at its base is inoculated with the test organism and incubated at optimum temperature for 24 hours. At the end of this time a new slant is inoculated from the condensation water. After 24 hours incubation of the second culture one or two drops of the condensation water (showing heavy growth) are poured aseptically into a large tube of sterile distilled water. Tubes having a diameter of one inch and containing 15–20 cc. distilled water offer a particular advantage here. These water cultures are incubated at optimum temperature for from 48 to 72 hours. As a rule, 72 hours are preferable to shorter periods.

After this incubation period a loopful of the water culture is transferred carefully to a clean glass slide. The loop is filled by dipping into the culture without stirring or shaking, and transferred to the slide by touching the latter without drawing the loop over the surface or disturbing the suspension more than is necessary, as flagella are broken off easily in this stage of the procedure. The drop on the slide is allowed to dry at room temperature or in a 37° incubator. No flame or heat higher than 37° is employed for drying.

After drying, the slide is ready to be placed in the mordant.

B. Preparation of mordant.

20% solution of tannic acid.....50cc.
made by moistening 20 grams, to reduce bulk, transferring to a 100 cc. flask and making up to volume with distilled water.

Cold saturated solution of ferrous sulphate in distilled water .....25cc.

Saturated 95% alcoholic solution of basic fuchsin, Coleman and Bell's, Grubler's, or others of equal grade....5 cc.

The mixture is allowed to stand 18 to 24 hours. The precipitate can now be removed by centrifuging or filtering thru an asbestos filter. The mordant may be used at this time, but cleaner preparations are obtained if it is allowed to stand in a Coplin jar for several hours, or even over night. It is then used without re-centrifuging or re-filtering.

The prepared slide is held in this mordant for 15 minutes at room temperature (20–23°C). It is then washed carefully in a slow stream of running water. After shaking off the excess water the slide is ready for final staining.

C. Preparation of stain.

Freshly filtered saturated solution of anilin water....100 cc.

Saturated 95% alcoholic solution of basic fuchsin.....12 cc.

[1] F. Loeffler, 1890, Weitere Untersuchungen ueber die Beizung und Färbung der Geisseln bei den Bakterien. Cent. f. Bakt., 7, 625–639.

These solutions are mixed and allowed to stand 18 to 24 hours, then centrifuged or filtered and transferred to Coplin staining jars.

After the mordant treatment, the slide is placed in this staining solution and kept there for 15 minutes at room temperature.

Then it is washed as before. The precipitate adhering to the underside of the slide may be wiped off. After drying the preparation is ready for microscopic examination.

Ziehl-Neelson carbol-fuchsin has been tried out simultaneously with the above staining solution, but has not given as good results as the anilin fuchsin.

Certain points should be emphasized in the microscopic observation of a flagella stain preparation. Of course, the highest magnification possible should be used for direct observation, the oil-immersion lens being indespensable. Care should be exercised to see that lens and ocular are clean.

Good light is necessary; this is best secured by artificial lighting of the field. It goes without saying that not every organism on the slide will show flagella. Quite often the only cells which show flagella are those which appear around the edge of the dried drop. A careful search of this region should be made before considering the preparation a failure. No explanation can be given by the writer for the variation in the intensity of stain in the flagella.

This method has given excellent results in a rather large number of trials in which members of the colon-typhoid-paratyphoid, the proteus, and the pseudomonas groups, and some members of the subtilis group, particularly *Bacillus cereus*, were employed. It is not an exaggeration to say that flagella stains of these organisms have been made in almost 100% of the trials made in this laboratory by the writer and by students in the course in general bacteriology when directions were followed explicitly.

We have been unsuccessful in securing flagella stains with organisms of the cholera and prodigiosus types or with *Bacillus megatherium* and a few other species. The writer hopes that some other investigator may be stimulated by the results reported here to further investigation which may lead to a method that will give consistently good results with all flagellated organisms.

In the carrying out of the method reported here, two points are of particular importance. First, for the groups of organisms mentioned, no short cuts should be attempted and full attention must be given to details, and second, heat must not be employed at any stage of the process.

The author wishes to express gratitude to Prof. L. F. Rettger for correction of this manuscript.

NOTES ON THE GRAM STAIN. II.

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ABSTRACT.—**The earlier investigations of the senior author have been continued, and the writers come to the following conclusions:**

1. The pH of the environment in which organisms are grown may affect the Gram reaction.

2. Organisms should be classed as Gram positive or Gram negative according to the staining reaction when grown in the optimum pH range for the species. Since the reaction may change with growth the media should be strongly buffered.

3. An alkali should be incorporated in the Gram staining technic in order to nullify the effect of an acid environment. We frequently wish to stain organisms from an environment not subject to control.

4. If alkali is added to the staining technic and the best American dyes are used the reaction of the medium upon which organisms are grown should not affect the Gram reaction.

5. There is no relation between acid and alkali production and the classification of bacteria regarding the Gram reaction. Acid and alkali producers are found in both groups.

6. The selection of a Gram staining technic should be based upon reliability of the methods under unfavorable conditions of acidity as well as on results obtained with borderline organisms.

There is included a discussion of the requirements of a practical Gram stain.

The effect of the hydrogen ion concentration on the Gram reaction has been well established. To eliminate the effect of an acid reaction Burke recommended the addition of a solution of sodium bicarbonate to the dye on the slide.[1] Sheppe and Constable[2] found that Grams iodine frequently becomes acid upon exposure to light and heat and that such increased acidity interferes with the Gram reaction. To eliminate the effect of an acid iodine solution Kilduffe[3] recommends the addition of sodium bicarbonate to Grams iodine.

[1]Burke, Victor, Notes on the Gram Stain with Description of a New Method. Jr. Bact. **7**, 159. 1922. The Gram Stain in the Diagnosis of Chronic Gonorrhea. J. A. M. A. **77**, 1020. 1921.

[2]Sheppe, Wm. M. and Constable, Mary G. An Unsuspected Source of Error in the Gram Stain. Jr. Lab. & Clin. Med. **8**, 575. 1925,

[3]Kilduffe, Robert A. A new Alkaline, Solution of Iodin for Use in the Gram Stain. J. A. M. A. **81**, 2182. 1923.

Kopeloff and Beerman[4] recommend the addition of sodium bicarbonate to the methyl violet and sodium hydroxide to the iodine solution. They report satisfactory results in the staining of milk smears which are usually acid. Stearns and Stearns,[5] attempt to explain the action of acid on the Gram reaction. They also point out that oxidation and reduction greatly influence the Gram reaction.

The effect of the acidity of the environment as well as of the staining solutions on the Gram reaction should be taken into consideration in the study of pure cultures and in the diagnosis of clinical material. The effect of acidity of the environment is readily avoided or eliminated. Pure cultures can be grown on neutral buffered media. Sodium bicarbonate can be added in the staining technic when the environment in which the organism grows is not subject to control. Burke has shown that sodium bicarbonate added to the dye on the smear eliminates the effect of a previous treatment with acid. The optimum reaction for the growing of bacteria varies with the different species. Since sodium bicarbonate eliminates the effect of a previous exposure to acid it appears more desirable to add sodium bicarbonate in the staining technic than to grow the organisms on media not titrated to optimum growth conditions. This conclusion is strengthened when we consider that we must occasionally stain organisms from an acid environment and that one staining technic should serve for all purposes.

The possibility of reducing the number of organisms variable to the Gram reaction by controlling the reaction of the medium was not determined. If the number can not be reduced by the addition of alkali to the staining technic it probably can not be reduced by modifying the reaction of the medium upon which the organisms are grown.

Experimental

Experiment 1. To determine the effect of the reaction of the media on the Gram reaction of Staphylococcus aureus.

Staphylococcus aureus was grown on gelatin infusion agar slants titrated to +1, +2, +3, +4, +5, +6 and —1, —2, —3, —4, —5, —6 to phenolphthalein. Smears were made in distilled water and stained by the Burke method (see footnote 1, p 63), with the exception that the sodium bicarbonate solution was omitted. The smears were made in triplicate and stained with 3 different samples of

[4]Kopeloff, Nicholas and Beerman, Philip. Modified Gram Stains. Jr. Inf. Dis. **31**, 480. 1922.

[5]Stearns, Esther Wagner, and Stearns, Allen Edwin. The Chemical Mechanism of Bacterial Behavior. 1. Behavior toward Dyes—Factors Controlling the Gram Reaction. Jr. Bact. **9**, 463. 1924.

gentian violet hereafter designated as I, II, and III. The smears were made mainly at 48 hour intervals up to 30 days.

The organisms did not grow well on the more acid and alkaline media. A heavy inoculation was necessary for growth and growth was meagre. The death rate among the organisms was probably high. It is likely that the organisms that grew were not subjected to the full effects of the reaction of the agar. Changes in the reaction of the media due to bacterial growth were not determined.

The stained smears were examined and classed as poor, medium and strong. Of the smears classed as poor, 46 were from acid media and 26 from alkaline media. Of the smears classed as strong 57 were from alkaline media and 24 from acid media. Of smears classed as poor 54 were found to be stained by gentian violet I, 14 by gentian violet II and 4 by gentian violet III. Of the smears classed as good 11 were stained by gentian violet I, 63 by gentian violet II and 8 by gentian violet III. Most culture media are titrated to a range between + 2 and — 2. Of the smears made from media within this range 56 were classed as strong and 39 as poor. Forty of those classed as strong and 18 of those classed as poor were from the alkaline media. Poor stains were obtained mainly after the first day or from the media adjusted to the extreme reactions. From the third day on, poor stains appeared with greater frequency but largely from the acid media. The organisms retained normal staining longer on the alkaline media.

The results obtained in this experiment suggest that the reaction of the medium upon which organisms are grown may effect the Gram reaction. Whether this effect is more pronounced when the organisms are grown in broth was not determined. The effect is more evident when the poorer dyes are used. Since the effect of acid on the Gram reaction can be eliminated by the use of alkali, as determined by the previous experiments of one of us and by an occasional test in this series of experiments, it appears advisable to incorporate sodium bicarbonate in the Gram staining solutions. It is our opinion that if this is done and the best dyes are used the effect of an acid medium or environment on the Gram reaction will be negligible. To standardize a method of determining the Gram reaction of bacteria we recommend that the organisms be grown on a strongly buffered solid medium titrated to the optimum pH for the species and that an alkali be incorporated in the staining technic.

It should be noted that our experiments deal with a Gram positive organism, and the undesirable effect of the environment caused it to become Gram negative. In view of the work of Stearns and Stearns we must keep in mind the possibility that an alkaline environment

may cause Gram negative organisms to become Gram positive. We have made no attempt to determine whether Gram negative organisms will grow or are ever found in an environment sufficiently alkaline to cause them to stain Gram positive. It has been our experience that the confusion concerning the Gram stain is due to Gram positive organisms becoming Gram negative rather than the reverse. It appears to us that Gram positive bacteria are more likely to become Gram negative due to acid production than Gram negative organisms are to become Gram positive due to alkali production.

Practical Gram Staining

For the differential staining of bacteria used in the class room or encountered in the diagnostic laboratory it is desirable that a reliable Gram staining technic be adopted. The staining technic best suited for this purpose may not be identical with the technic best suited for the study of certain other organisms or to be used in the description of new species. One technic may be superior for the study of organisms in pure culture but inferior for the study of organisms in acid pus. The important thing is not that a certain organism is listed as Gram positive, Gram amphophile or Gram negative but that we have a staining technic that will place it with considerable certainty and under a wide variety of conditions in one of the three groups. Those of us interested in diagnosis are not interested in a technic that will class an organism as Gram positive when grown on media of a certain reaction but will not so classify it when found in acid pus. We are not interested in a technic that will cause a few more of the Gram amphophile organisms to become Gram positive under special conditions unless it is more likely than any other technic to cause them to stain Gram positive under all the conditions in which we wish to stain them. Therefore we suggest to those interested in sifting out the most reliable Gram staining technic to pay special attention to the reliability of the various methods under unfavorable conditions.

It is desirable that we have a Gram staining technic that is easily mastered by the novice, that will differentially stain bacteria irrespective of the age of the organisms or of the acidity and nature of the environment; that is rapid; that will give satisfactory results with the different makes of dyes; and that requires the use of solutions that are stable. With these requirements in mind, we recommend that an alkali be incorporated into the staining technic and that acetone be used as the decolorizer. It has been shown that an alkali neutralizes the interfering effect of a previous exposure

to acid and that it causes old cultures to stain more typically.[6] The kind of alkali and method of application is subject to choice.[7] Not all alkalies can be used.

It has been our experience that acetone is a better decolorizer than alcohol, as the former is more rapid, is less likely to decolorize the Gram positive organisms when properly used and can be depended upon to decolorize the Gram negative organisms.[6] It is especially advantageous when there is much extraneous matter on the slide. Elementary students master the use of acetone as a decolorizer more readily than the use of alcohol. Following the addition of alkali to the staining technic and the use of acetone as the decolorizer the Gram stain has ceased to be a problem among the students in this laboratory when working with unknown organisms. Before that the Gram stain was always a problem.

A Japan Drier manufactured by the Bass-Heuter Paint Company was found to serve as a satisfactory substitute for acetone. This Drier has an odor of acetone but is described in a letter from the company as a "combination of Rosin, Linseed Oil, lead and manganese oxide driers reduced with a hydrocarbon spirit." The active agent was not determined.

Hucker & Conn,[8] in comparing alcohol and acetone as decolorizers state that acetone does not give as constant results as alcohol. They further state that more data are needed before definite conclusions are drawn. We are of the opinion that the technic used by them can be modified in such a manner as to show a decided superiority for acetone over alcohol as a decolorizer. It may not, however, prove more satisfactory in the study of pure cultures under certain controlled conditions of environment.

[6]See footnote 1, p. 63.
[7]See footnotes 1, 2, and 3, p. 63.
[8]Hucker, G. J. and Conn, H. J. Methods of Gram Staining. N. Y. Agr. Exp. Sta., Tech. Bul. 93. 1923.

MODIFICATION OF THE FRENCH AZURE C TISSUE STAIN

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Since azure is taken out of the nuclei very rapidly by alcohol in tissues fixed in other solutions than formalin, some difficulty has been experienced in using the French technic[1] following most fixatives. Inasmuch as formalin shrinks tissues and is not a delicate fixative (tho useful for quick work), the modifiçation here suggested may be of value as adapting the azure technic to finer fixed tissues.

In this modification, clove oil is used as a medium for dissolving the counterstain, since it takes out azure from the nuclei much more slowly than alcohol. A saturated solution of alcohol soluble eosin in clove oil makes a very good counterstain, as this may be followed directly by xylol. French's mixture of eosin Y and orange II may be used as a counterstain, if preferred, in a saturated clove oil solution; but as xylol precipitates these dyes, the sections must be run thru clove oil 10–30 seconds after counterstaining, before placing in xylol. In the modification recommended, the azure C specified by French, which is not yet commercially available, may be replaced by the better known methylene azure (azure I).

French's Method	*Modification*
1. Xylol 3 mins.	1. Xylol 3 mins.
2. Abs. Ethyl Alcohol 3 mins.	2. Abs. Ethyl Alcohol 3 mins.
3. 95% Ethyl Alcohol 3 mins.	3. 95% Ethyl Alcohol 3 mins.
4. Water 3 mins.	4. Water 3 mins.
5. 1.5% aqu. sol. Azure C, 5 mins.	5. 1.5% aqu. sol. methylene azure (Azure I), 5 mins.
6. Abs. Methyl Alcohol 5–10 sec.	6. Abs. Ethyl Alcohol 5–10 sec.[2]
7. Eosin Y-Orange II, .05gms. Glacial Acetic Acid 1.0 cc. Abs. Ethyl Alcohol 99. cc. 30–40 sec.	7. Sat. sol. of Alcohol soluble Eosin[3] in Clove Oil 30 secs.
8. Abs. Alcohol 1–2 mins.	8. Xylol 10–30 secs.

[1]Holmes, W. C. & French, R. W. The oxidation products of Methylene Blue. Stain Technology, 1, 17–26.

[2]For formalin fixed material French suggests this modification except that 3% glacial acetic in absolute alcohol be substituted for plain absolute alcohol in step No. 6.

[3]By alcohol soluble eosin is meant ethyl eosin (C. I. No. 770). This is not yet on the market in this country; but an experimental batch has been prepared by the National Aniline and Chemical Co., and will soon be available.—Ed.

9. Abs. Alcohol 1–2 mins.	9. Xylol 1–2 mins.
10. Xylol 1–2 mins.	10. Xylol 1–2 mins.
11. Xylol 1–2 mins.	11. Xylol balsam
12. Xylol balsam	

This technic has been tried with picro-sulphuric, Bouin, Zenker, and 95% alcohol—3% bichromate as fixatives. The solution of the counterstain in clove oil may be used repeatedly thus cutting down the item of expense. Slides have been sent to French who regards this modification as an improvement over the original technic.

AN AIR DISTRIBUTER FOR USE IN THE DROP METHOD OF DEHYDRATION

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The superiority of results as well as the great convenience of raising fixed tissues through alcohol and oils drop by drop has been recognized for several years. As the various fluids involved in the processes leading to imbedding do not mix readily it is necessary to have some means of agitating them. Air pressure which is led through the solutions as a slow stream of bubbles is usually the most available and adaptable source of energy. Various devices for producing a flow of air have been described.[1] With most of these, it has, in general, been possible to supply air to but one or two vials at a time. Where a constant air pressure is available and it is necessary to dehydrate a large number of tissue samples at one time an apparatus for distributing the air as shown in the accompanying sketch is useful.

The apparatus consists of a central reservoir (Fig. 1-b) which is elevated on a block of wood and which is kept filled by the slow flow of liquid from the large bottle above (a). This reservoir is placed in a vessel 8 to 12 inches in diameter and is surrounded by the vials (d) containing the tissue to be dehydrated. A series of glass Ts (e) joined with short pieces of rubber tubing is wired in place around the outer edge of the large vessel. One of these Ts (f) is connected to the main supply of air pressure, while from the others rubber tubing leads the air into the vials of tissue. Each tube has its own screw clamp for controling the quantity of air that flows through the liquid. The alcohol or oil passes from the reservoir (b) through capillary glass siphons (c) into the surrounding vials. The siphons are readily made from capillary tubes that are drawn from tubing of large bore. They are bent to the correct shape by passing the small tubes rather quickly in and out of a small flame while maintaining a slight pressure in the direction of the desired bend. It is useful roughly to calibrate the finished siphons and to keep them separated according to the number of drops they deliver per minute (g). The details of the process have been described by Allen.

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[1]Allen, Ezra. 1916 Studies on cell division in the albino rat. II Anatomical Record **10**, p. 585–589.
Hance, Robert T. 1925. Notes on technique from the University of Pennsylvania. Trans. Amer. Mic. Soc. 1925 p. 162–167.
Painter, T. S. 1924. A technique for the study of mammalian chromosomes. Anatomical Record **27**, p. 77–87.

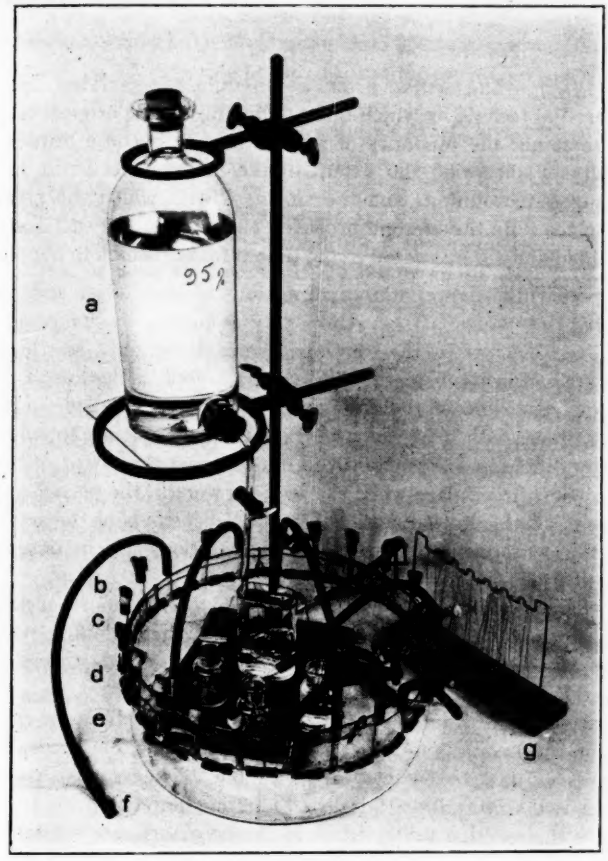

FIG. 1. Photograph of air distributer for use in the drop method of dehydration.

a. Main reservoir of alcohol or oil that supplies the small reservoir,
b. from which capillary siphons,
c. lead the liquid drop by drop from b to—
d. the tissue vials.
e. circle of glass Ts used as air outlets. These are wired in place around the dish. When not needed they are corked with short lengths of rubber tubing which have their upper ends bent over and tightly taped down. In use 6–8 inch pieces of rubber tubing are slipped on to as many outlets as needed and led through pointed glass tips into the tissue vials. The volume of air is controled with individual screw clamps.
f. tubing that connects the circle of outlets with the main air supply.
g. rack for glass siphons c.

THE WEIGERT-PAL TECHNIC FOR STAINING MYELIN

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ABSTRACT.—**The writer gives a schedule for carrying out the Weigert-Pal technic by which three difficulties of the original technic are overcome: the tendency of the sections to become brittle; the difficulty of observing the extent of the reaction occurring in the permanganate solution; and the slowness with which the reaction takes place. By the method proposed as many as 300 sections may be stained and differentiated in the time it formerly took to handle 50.**

In preparing a large number of sections of brain stem and spinal cord by the Weigert-Pal method, it was found, by following the usual technic,[1] that the sections were exceedingly brittle after being stained in the hematoxylin. This resulted in the loss of many sections, regardless of how carefully they were handled, in transferring them with a section lifter from one solution to another. Another difficulty was encountered in observing the extent of the reaction which took place when the sections were in the permanganate solution. A third disadvantage was in the slowness of the action of both the permanganate and oxalic acid solutions which consumed a great deal of time for differentiation.

By leaving the sections in 80% alcohol for 12 hours or longer, after they had been stained in hematoxylin and before differentiating, it was found that they were much less brittle and also were more easily differentiated.

In the differentiation process we used large Petri dishes or crystallization dishes containing fluid to a depth of not over one inch. These were placed on a box with a top of double thickness glass, and containing two white 50 watt bulbs. This not only permitted one to observe the reaction taking place in the permanganate solution, but also supplied enough heat to accelerate the action of the potassium permanganate and the oxalic acid.

It was also found that sections cleared in oil of bergamot lost their sharp colors in a few months whereas those cleared in origanum oil and carbol xylol have remained in good condition for over three years.

The schedule we used was as follows:—

1. Formalin fixed material is embedded in celloidin and sections 75–90 microns are cut. Sections of this thickness show the fiber tracts very well.
2. Mordant in Müller's fluid in a warm place, for 12–18 hours. A paraffin oven at 35–39° C. is best.

[1]See: Guyer, Animal Micrology, 2nd Ed. p. 236, and Lee, Microtomist's Vademecum, 8th Ed. p. 446.

3. Wash several times in distilled water.
4. Stain 8–24 hours in Weigert-Pal hematoxylin made as follows:
   10 cc. of 10% ripe hematoxylin solution in absolute alcohol.
   90 cc. of distilled water.
   1 cc. of 1 part saturated solution of lithium carbonate to 80 parts of distilled water.
5. Wash in distilled water with a few drops (6 to 8 to 1000 cc. water) of lithium carbonate for 3–5 minutes.
6. Place sections in 50% alcohol for a few minutes.
7. Then in 80% alcohol for 10–12 hours. This step makes the sections less brittle and the differentiation easier.
8. Pass back through 50% alcohol to water.

In the following steps of differentiation two large Petri dishes containing the permanganate and oxalic acid solutions and a large crystallization dish containing distilled water are placed on the improvised glass hot plate, which illuminates the potassium permanganate solution and warms both the permanganate and oxalic acid solutions.

9. Place sections in a 0.25% aqueous solution of potassium permanganate until brown (½ to 3 minutes). The gray matter can be seen through the illuminated solution as a light brown.
10. Rinse in distilled water 1–3 minutes.
11. Place in a freshly prepared solution of equal parts of a 1% aqueous solution of oxalic acid and a 1% aqueous solution of potassium sulphite, to remove the brown color. This should leave the myelin a dark blue to black color and the gray matter a pale gray or white. If all the brown color does not come out, repeat the treatment with permanganate solution a short time. The potassium sulphite must be fresh for good results.
12. Wash in 3 changes of distilled water.
13. Dehydrate through 50%, 80% and 95% alcohols.
14. Clear in origanum oil or better carbol xylol.
15. Mount in neutral balsam.

By this method as many as 300 sections may be stained and differentiated in the time it usually took to handle 50.

CHEMICAL STRUCTURE AS AFFECTING THE STAINING AND THERAPEUTIC PROPERTIES OF DYES

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ABSTRACT.—**Toxicity resulting from the oxidation of methyl groups in certain therapeutic dyes seems to occur as the result of formaldehyde produced. It is suggested that ethylation be employed in lieu of the methylation to counteract this toxicity. In the triphenylmethane dyes it is found that apparent differences in staining and bactericidal value may possibly be accounted for by differences in structure, a rosaniline base in general being superior to the pararosaniline compounds.**

Thru the employment of the spectrophotometer in the study of therapeutic dyes of the triphenylmethane series (gentian violets) in the blood stream and bile, following intravenous administration, it has been noted that a progressive oxidation of alkyl groups occurs.[2] Oxidation of methyl groups, such as are ordinarily found in the gentian violets, results in the production of formaldehyde, which in turn is readily oxidized into formic acid within the system. This oxidation occurs rapidly and at least two and probably three alkyl groups are split off in the blood stream within a short time after administration of the drug.

This formaldehyde and resultant formic acid should result in considerable pathological effect in the kidney and study shows this to be true. Furthermore, it would be expected that such agents injected subcutaneously would result in more or less tissue necrosis due to the formaldehyde produced. This also is true.

Similar toxic effects are to be anticipated in the employment of methylene blue (tetramethyl thionin) in which the methyl groups also are readily oxidized. It also is to be expected with any dye carrying readily oxidizable methyl groups.

To overcome this inherent fault with certain dyestuffs proposed for therapeutic application, it is suggested that ethylation be employed in lieu of methylation, as the oxidation of ethyl groups will result in the production of ethyl aldehyde which is much less toxic than formaldehyde or the resulting formic acid. It also is the writer's opinion that in many instances compounds with fewer substituted amino hydrogens may be employed as effectively with consequently less resulting oxidation with deleterious products.

[1]Contribution from the Laboratory Service, Walter Reed General Hospital, Army Medical Center, Washington, D. C.

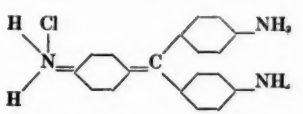
[2]Summary of work on Chemotherapy at Army Medical Center. Awaiting publication.

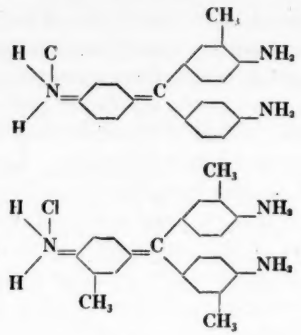
In studying the germicidal value *in vitro* of acriviolet (a mixture of neutral acriflavine and gentian violet) examination was made, jointly with Dr. G. F. Reddish, U. S. Department of Agriculture, of the components alone, as well as of a number of gentian violets of domestic and foreign origin, at first it appeared that degree of alkylation was a factor in relative bactericidal value. Later, however, it became apparent that another factor is of even greater import with regard to the alkylated triphenylmethane dyestuffs as will be discussed below. The results of this study tend to show that an effective germicidal value may be obtained in a dye with the substitution of three or four of the amino hydrogens by alkyl groups in triphenylmethanes. Study also has shown that ethylation is fully as effective in this regard as methylation.

Still other considerations are brought out in dealing with the methyl and crystal violets as staining and therapeutic agents. First, as regards stain effect in the Gram technic, it has been noted since the World War that certain samples of methyl violet are satisfactory others entirely unsatisfactory. Some of this trouble has been traced to acid formation in the iodine solution, but quite a little of the trouble has been definitely due to little understood variations in the violets. In general, crystal violet is entirely satisfactory.

The violets, chemically are formed by the replacement of the amino hydrogen in the basic fuchsins by alkyl groups—commonly methyl. For many years many were produced by the direct methylation of fuchsin, but later have been manufactured by direct synthesis. This has been specially true with the higher methylated homologues as crystal violet (hexa-methyl para-rosanilin) and penta-methyl para-rosanilin. Direct methylation has been almost entirely superceded by the methods of synthesis. An outstanding feature of this change in manufacture is the fact that practically all violets of this group are para-rosanilins, while heretofore they consisted of a mixture of methylated rosanilin and para-rosanilin hydrochlorides.

This difference can be understood by recalling the formulae of the three primary basic fuchsins, namely rosanilin, para-rosanilin and new fuchsin. Basicly they have the three structural formulae:





In passing from para-rosanilin to new fuchsin, an increasing degree of methyl substitution is noted in the meta position to the methane carbon.

In practice the older methyl violets, often of a very low degree of methylation, (in the amino groups), performed satisfactorily in the Gram stain. The more recent products are doubtful in performance. This likewise is believed to be explainable by this basic difference in constitution. It seems as tho the rosanilin structure favors chemical combination, while the para-rosanilin does not. This is specially noticeable in lower methylated products, in which amino hydrogens remain free for substitution; and does not appear to apply in the case of crystal violet in which all amino hydrogens have been substituted.

Another field in which some of these differences are particularly noticeable is in the application of certain triphenylmethane dyes as therapeutic agents. Here marked variations in apparent potency are found in the various samples of methyl and gentian violets. At first it appeared, as mentioned above, that degree of alkylation affected the bactericidal value, a crystal violet being found to be more effective than a lower methylated homolog. To verify this, samples of methyl violets of varying degrees of methylation were prepared. No marked variation comparable to degree of alkylation was noted. Also a sample of pre-war gentian violet which was of low alkylation and very much of a mixture, but which is regarded as largely a methylated rosanilin, proved to have several times the bactericidal value of other similar dyes.

Hence, it seems probable that a rosanilin is preferable as a possible therapeutic agent; also it is believed that this may be the real difference between many pre-war and present samples of gentian violet.

In biological use there is another interesting fact in the staining of the so-called "acid-fast" organisms. These bacteria are difficult to stain, but once stained resist decoloration with acids. There also are a group of organisms known as spore-bearers in which certain resistant spores are formed. These, too, are difficult to stain. For the staining of these objects it has been common practice to use either basic fuchsin or crystal violet in conjunction with phenol.

Ziehl-Neelson carbol fuchsin has been employed for many years as a stain, dating from 1882, and it has been generally supposed that the phenol acts as a mordant. If this were true, it would be reasonable to assume that it could be used separately, preceding the fuchsin. Trial shows that such is not the case. From this test it seems logical to assume that there is a definite combination between the phenol and fuchsin to form a phenylated fuchsin. If that is the case, such a dye should be possible of isolation.

On these assumptions a basic fuchsin (mixture of rosanilin and para-rosanilin hydrochlorides) was refluxed with alcohol, water and phenol. The resulting product was freed of excess phenol by steam distillation, the solution acidified and the dye recovered by salting out as the hydrochloride. Upon trial, it was found that the resulting dye in simple aqueous solution acted the same, so far as stain action is concerned, as the customary carbol fuchsin.[3]

Spectrophotometrically the phenylated rosanilin shows little change in its absorption curve from that of the original dye.

In resume, it seems logical to assume that chemical structure of the dye plays an important role in its performance either as a staining agent or as a bactericide and that the bactericidal effect often closely parallels the staining action.

[3]Patents pending. Following up this basic work it has been found that the synthesis of basic dyes with cyclic derivatives containing acidic or phenolic groups is easily accomplished and common between the two compounds.

NOTES ON TECHNIC

A Quick Iron-Hematoxylin Method. Without doubt iron-hematoxylin as developed by Heidenhain is one of the clearest and most useful stains, bringing out structures with greatest intensity and sharpness. Its one drawback has been the length of time required for the procedure. During the war, while working on intestinal protozoa, it was found that no stain could be substituted for iron-hematoxylin for making permanent records of fecal smears. It was, therefore, essential to devise a rapid method for handling hundreds of slides and making rapid diagnosis. The following methods were adopted after many experiments:

A small part of the feces was rubbed up on a clean slide in physiological salt solution and examined under the microscope for active protozoa. Then a drop of Donaldson's iodine-eosin stain was added to bring out the cysts. This concluded the preliminary examination, and if the case were positive, four permanent slides had to be made for confirmation and as a matter of record. Four clean slides were taken and numbered with a diamond to correspond to the patient's number in our daily record. A clean applicator stick was then used to obtain material which contained either mucus or blood, if such was present, and streaked lightly on the slide. The slides were then dipped without drying into Coplin jars of hot Schaudinn's fluid, left there 5 minutes, washed in 50% alcohol, then 50% alcohol plus iodine to remove any bichloride, 35% alcohol, then water. The wet slides were then placed on an electric hot plate and a 4% iron alum solution was dropped on until the slides were covered and steaming. Any evaporation was taken care of by the further addition of iron alum from a pipette. This was continued for several minutes. Slides were then rinsed in water, put back on the hot plate and a ripe 0.5% hematoxylin solution was added by means of a pipette, the slide being kept full of liquid and steaming all the while. After several minutes, they were placed in water, differentiated in cold 2% iron alum solution, controlling the decolorization from time to time under the microscope. When the nuclei in the best parts of the smear stood out nicely, decolorization was stopped and the slides washed in running water for at least 5 minutes. They were then dehydrated and mounted in balsam in the usual manner.

Thus within a half hour it was possible to have finished permanent slides with fine cytological details shown to their best advantage.

These preparations are now about 7 years old and they are still as good as they were originally.

It might be added that our experience with hematoxylin showed that black crystals would not do for this work, but that the white or light brown crystals made up in 10% solution in alcohol and ripened and then added to water to make up 0.5% hematoxylin would work nicely in our process.—S. I. Kornhauser, Louisville, Kentucky.

FAT STAINS. Cooperative study of fat stains is being undertaken by the Army Medical Museum and St. Elizabeths Hospital, and certain results to date show that certain commercial dyes are superior to specially purified Sudan III (C. I. 248), of which several samples have been examined. Two commercial products have been found to be specially meritorious, Candle Scarlet (Geigy) and Oil Red O (National Aniline). Both have a greater depth of color than the usual Sudan III; and of the two Oil Red O is superior in this respect. It is understood that the technical products are composed of a mixture of isomers, whereas the purified Sudan III is a single isomer. —R. W. French, Washington, D. C.

AZURE C TISSUE STAIN. In employing The Azure C, Eosin Y, Orange II tissue stain given in the first issue of this JOURNAL,[1] it has been found that the strength of the acetic alcohol may be increased to 3 per cent. glacial acetic acid in absolute alcohol with a greater certainty and ease in differentiation. This strength of acid will not decolorize the nuclear or bacterial elements appreciably.

It also has been noted that the present samples of Azure C available do not work satisfactorily when first prepared in solution, but improve with age. This may be overcome, however, by employing the following combination of Azure C and Methylene Violet (Bernthsen):

Azure C	1.5 gm.
Methylene Violet	.1 gm.
Ethyl alcohol, 95%	25.0 c.c.
Distilled water	75.0 c.c.

Azure C is being prepared by synthetic methods and with a purer product available the original formula is entirely satisfactory. While theoretically possible, it is believed impracticable to prepare azures of sufficient purity by the de-alkylation of Methylene Blue.

[1]Holmes, W. C. & French, R. W. The Oxidation Products of Methylene Blue. Stain Technology, 1, p. 11–16.

If reasonable care is employed, step No. 6 calling for absolute methyl alcohol may be changed to a step of 3 per cent. glacial acetic acid in absolute ethyl alcohol. This affords excellent differentiation and aids materially in prolonging the life of the counterstain mixture—R. W. French, Washington, D. C.